SURFACE STERILIZATION OF TISSUES FOR BACTERIAL STUDIES

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In a series of experiments for the purpose of studying the bacterial content of the prostate, certain difficulties were encountered with the methods commonly described for making cultures from tissues. It was found that by using the method recommended by Rosenow[1] certain discrepancies occurred in the results which suggested that they may have been due to contamination introduced at some point between the time the tissue was exposed by the surgical scalpel and the final closure of the culture tube. In order to decide this question decisively it was determined to put a close check on each step in this process.

Within the last few years a great deal of interest has been taken in the bacteriology of glandular tissues and its connection with the etiology of disease. Negri and Mieremet[2] and Bunting and Yates[3] almost simultaneously isolated a pleomorphic diphtheroid bacillus from the glands in Hodgkin's disease. Later Billings and Rosenow[4] were also successful in cultivating a diphtheroid bacillus in this condition. Rosenow,[5] Rhea and Falconer,[6] Bloomfield,[7] Torry,[8] Langford,[9] Cunningham,[10] Wade and Harris,[11] Fox[12] and others have also reported the recovery from Hodgkin's disease of several different types of diphtheroid organisms, pigmented and nonpigmented, and associated with these they found various organisms as contaminators; e. g., staphylococcus albus and aureus, streptococcus, B. welchii and others. Bunting and Yates[3] say that "although the utmost efforts were made to prevent carrying in organisms from the skin when removing nodes for cultural investigation, in almost every case studied, one or more tubes have shown the presence of a white staphylococcus." Torry[8] in a study of normal and abnormal lymph nodes found a diphtheroid bacillus of one type or another from 22 of 40 cases in such

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[1] Centralbl. f. Bacteriol., I, O., 1914, 74, p. 366; Jour. Am. Med. Assn., 1914, 63, p. 903.
[2] Centralbl. f. Bacteriol., I, O., 1913, 67, 292.
[3] Arch. Int. Med., 1913, 12, p. 236; Jour. Am. Med. Assn., 1913, 61, p. 1803; ibid., 1914, 62, p. 516; ibid., 1914, 62, p. 177.
[4] Jour. Am. Med. Assn., 1913, 61, p. 2122.
[5] Jour. Am. Med. Assn., 1914, 63, p. 903.
[6] Arch. Int. Med., 1915, 15, p. 438.
[7] Arch. Int. Med., 1915, 16, p. 197.
[8] Jour. Med. Res., 1916, 29, p. 65.
[9] Am. Jour. Trop. Dis. and Prev. Med., 1914, 2, p. 191.
[10] The Am. Jour. Med. Sc., 1917, 153, p. 406.
[11] Jour. Exp. Med., 1915, 21, p. 493.
[12] Arch. Int. Med., 1916, 16, p. 465.

divers pathologic conditions as Hodgkin's disease, chronic hyperplastic lymphangitis of obscure nature, lymphosarcoma, sarcoma, melanoma, endothelioma, tuberculous adenitis and chronic lymphatic leukemia. The organisms were studied and grouped. Animal inoculations with these organisms were made in monkeys and the results were negative. Again, he recovered as many as 5 distinct types of diphtheroids in a single case of Hodgkin's disease. Tests made for agglutinins were negative. In conclusion, he says "cultural findings in various types of abnormal gland have indicated that this bacillus does not stand in specific relationship to any definite pathologic condition." This is substantiated by agglutination and complement fixation experiments with the blood of the patients. As he has found a bacillus of such uniform type and occurring so frequently in abnormal states of lymph gland he suggests the name of B. lympophilus. Rosenow,[1, 13] of 54 cases of arthritis deformans, isolated a nonhemolyzing streptococcus 32 times, staphylococcus 5 times, B. welchii in 14 cases, an organism resembling B. mucosus 3 times, M. catarrhalis and the gonococcus once each, and diphtheroid bacillus in 5 cases; in 7 cases the cultures remained sterile. In 7 cases of erythema nodosum excised nodes from the cervical lymph glands gave a polymorphous, sometimes clubbed diplobacillus. Out of 32 cases of goiter in man, he isolated an anaerobic gram-positive diplobacillus-like organism from the thyroid gland. And in 8 out of 12 dogs having goiter B. welchii was found in all but 6 of the thyroid glands. A hemolytic staphylococcus was found in most goiters, in man and dog. In another report,[13] he obtained positive results from glands only 5 mm. in diameter. The number of colonies ranged from one to two thousand. In 38 cases he isolated organisms from all but 3 cases. Streptococcus was obtained in 14 cases. B. welchii was obtained in 9 cases, staphylococcus in 3 cases and gonococcus in one. Cunningham,[10] in his gland cultures obtained in some cases pure cultures of staphylococcus in some of the tubes. Finch[14] isolated a sporothrix from axillary glands.

A number of these investigators[8, 10, 12] have been unable to identify by serological reaction the organisms recovered by them with that described by Bunting and Yates. In addition Torry, Fox, Cunningham, and Cellar (as reported by Libman[15]), have found diphtheroid bacteria in tuberculous and other glands not the seat of Hodgkin's disease Harris and Wade[11] have shown that diphtheroids are widely distributed in nature, as they recovered them from the air, body surfaces, and at times from deep tissues so that they concluded that they were there through contamination or else they are indigenous in these locations. Ford[16] and Nicolles[17] have stated that they are present in normal tissues, and in pathologic conditions to which they bear no etiologic relation, as in lesions of leprosy, blastomycosis, tertiary syphilis, and tumors of various types, as did Torry, Cunningham and Cellar. Harris and Wade[11] also cite the fact that organisms have been isolated from human tissue, removed with presumably sterile precautions. Wolbach and Saike[18] isolated an anaerobic spore-bearing bacillus from apparently normal animals in 21 of 23 cases.

In spite of the great quantity of work done the divergent results still leave open the question as to the relations the organisms isolated bear to the diseases in question.

[13] Jour. Am. Med. Assn., 1914, 62, p. 1146.
[14] Proceedings New York Path. Soc., 1914, 14, p. 141.
[15] Jour. Am. Med. Assn., 1914, 63, p. 907.
[16] Trans. Assoc. of Am. Physicians, 1900, 15, p. 389.
[17] Canad. Jour. Med. and Surg., 1899, 6, p. 405.
[18] Jour. Med. Res., 1909, 21, p. 267.

SUMMARY OF PREVIOUS METHODS

Steele[19] grinds up inguinal glands in a sterile mortar but no further particulars are given. Langford[9] uses the bunsen flame. Fox[12] dips the tissue in hot oil (length of time not given), transfers it to ether, to salt solution and then to a large sterile bottle where it is cut up with scissors. Torry[8] sterilizes the surface by "dipping momentarily in boiling water and then transferring quickly to cool sterile saline solution. The gland is then placed in a sterile dish and macerated under a glass cover with fine scissors, not over five minutes elapsing, as a rule, during this procedure." Finch[14] "keeps the glands sterile," but does not describe how it is done. Rhea and Falconer[6] do not describe any method of sterilization. Bloomfield[7] washes the

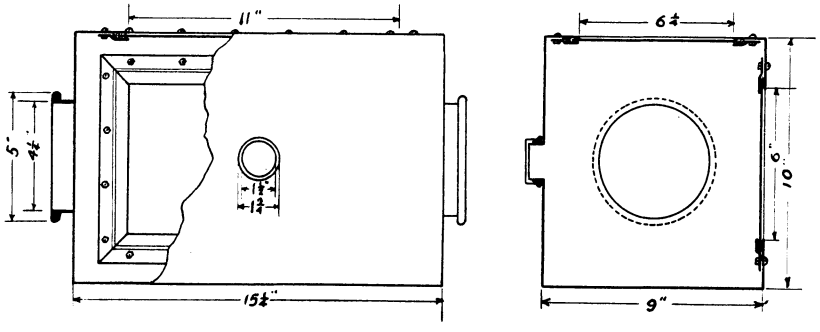

Fig. 1. Scale drawing of air chamber. Note construction of small hole for introduction of tissue. Edge of cap need not touch edge of hole, which is one-fourth inch smaller all around.

tissue several times in sterile salt solution, then dips it in boiling salt solution, the lengths of time varying with the size of the tissue. Rosenow[1] says, "Zur Sterilisierung der Aussenflächen werden dieselben durch eine Bunsenflamme gezogen odor für kurze oder längere Zeit in kochendes Wasser getaucht; der Zeitraum für die letztere Prozedur is von der Grösse der Gewebestückes abhängig, worauf die so sterilisierten Gewebe sofort in kalte, sterile Kochsalzlösung gelegt werden." In a later publication, [5, 20] he further states that he receives the tissue in gauze and carries it to the laboratory, emulsifies it, after stterilization, in broth or salt solution in a mortar in a specially devised sterile chamber. Cunningham[10] brought the glands to the laboratory in a sterile towel, 'mashed or squeezed' them and then placed them on

[19] Boston Med. and Surg. Jour., 1914, 170, p. 123.
[20] Jour. Inf. Dis., 1915, 16, p. 367.

various media. The instruments used were from the autopsy room, hastily boiled up. With this method various organisms which the author thinks were due to contamination, were obtained. Later improvements in the technic, the details of which are not specified, reduced the number of positive cultures. The only reference to the length of time used in surface sterilization made by any authors consulted is that it varied with the size of the tissues.

DESCRIPTION OF THE APPARATUS

Owing to the fact that the air chamber recommended by Rosenow as found on the market is not air-tight; that it is very unstable; that

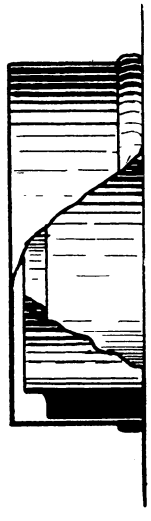

Fig. 2. Larger half section drawing of small hole for introduction of tissue.

only one hand can be introduced and that the large cotton plug used is difficult to manage, it was decided to design a new chamber to eliminate these difficulties. The chamber, as finally constructed, is rectangular and made of heavy tin with large panes of plate glass at both the top and front, a hand-hole at each end, to which canvas gloves are attached, so that both hands can work on the inside of the box, and an opening on the metal side for introducing the necessary materials and tissue. As can be seen by referring to the accompanying diagram, the flange of this opening is one-fourth inch smaller than the cap, so that the edge of the cap never comes in contact with the flange. This eliminates the cotton plug.

TESTS OF APPARATUS

In order to make sure that this apparatus accomplished the objects for which it was designed, tests were made, cultures of pigment-producing bacteria having been previously placed on the tissues and hands, according to the following scheme:

Tissue that has been autoclaved is cut into pieces of desired size, approximately 1.5 × 2 × 1.5 cm., in a sterile jar, dipped in a 24 hour broth culture of Staphylococcus aureus, and the methods of surface sterilization to be subsequently described carried out. One piece of

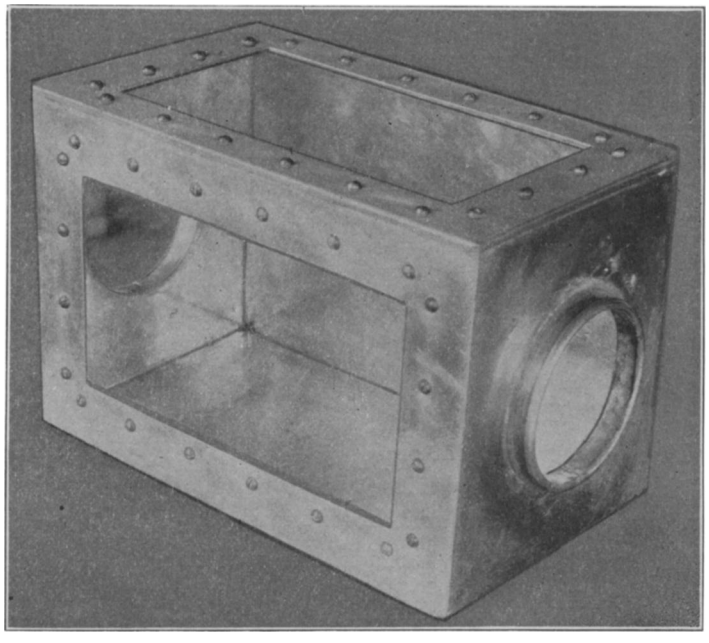

Fig. 3. Air chamber, showing glass windows and hand-holes.

the tissue is boiled 20 seconds in salt solution, another piece is flamed for 20 seconds, and a third piece is placed in oil at 180 C. for 5 seconds. The tissue is then dropped through the small opening into a mortar in the airchamber, to which a large test tube of dextrose ascitic broth[1] has previously been added (flaming the opening each time it is opened and closed). The chamber contains mortars, pestles, quartz sand, a block of wood holding four large test tubes, scissors and forceps, the whole having been sterilized for one hour at 160 C. each time before

use. The hands are washed for 10 minutes in soap and water, rinsed several times in bichlorid 1: 1000, then in distilled water and lastly in sterile salt solution. The hands now being nongermicidal and relatively sterile, are dipped in a broth emulsion of a 24 hour culture of B. violaceus and introduced into the sterile canvas gloves. The tissue is then cut up into very fine pieces, ground for 15 minutes, quartz sand is added and the grinding continued for 15 minutes longer. This emulsion is poured into one of the large test tubes which is then removed and after washing the hands plates of the emulsion are made, using 1 c.c. for each plate.

With this method all the plates remained sterile, neither Staph. aureus, B. violaceus nor any other organisms growing upon them.

Having demonstrated that the air chamber served to prevent contamination of the tissue, it was now necessary to check the method used for the surface sterilization of the pieces of tissue, which are usually obtained from the operating room. To this end, previously sterilized tissue inoculated on the surface or the interior with known organisms, was used.

OUTLINE OF METHOD

1. Mortars or evaporating dishes and pestles, scissors and tissue forceps are placed in a wire basket and sterilized in the hot air oven for one hour at 190 C., the mortars or dishes being upside down.

2. The tissue to be used, preferably kidney or liver, is autoclaved, placed in a sterile glass jar containing tissue forceps and scissors, and cut into pieces about 1.5x2x1.5 cm. in size.

3. A piece of tissue is dipped in an emulsion of a 24 hour broth culture of the organism for an instant; that is, just enough to cover the surface.[21]

4. The tissue is removed and placed in hot oil (liquid paraffin) boiling salt solution or a Bunsen flame the desired length of time.

5. It is dropped at once into the sterile mortar to which has been previously added 10 c.c. of sterile broth, and ground with the sterile

[21] It is understood that this is an exaggeration of the infection usually present on tissues to be cultured, but that such infection is not negligible is shown by a recent culture of 10 c.c. of broth into which a piece of prostate, dropped by the operator into a sterile glass jar, had been placed for a moment. A plate made with 1 c.c. contained 250 colonies. In another case, 1 c.c. of broth in which the prostate had been immersed produced 250 colonies, whereas after immediate surface sterilization for eight seconds in liquid paraffin at 180° C., emulsifying the tissue, in which a pus cavity was found, and plating 1 c.c. of this emulsion, the colonies were innumerable.

pestle, thus breaking up the tissue. The air chamber was used in the first few series but was deemed unnecessary for these thermal experiments.

6. One c.c. of this tissue emulsion is removed and plated. Repeat this procedure, changing the time of heating and media as desired.

7. As a check on the tissue used, a piece which has not been inoculated is emulsified and 1 c.c. of this emulsion is plated.

8. As a check on the organism and to have a culture where no sterilizing methods had been used with which to compare the growth

TABLE 1

TESTS WITH BOILING 0.85 PER CENT. NaCl SOLUTION

Tissue Inoculation* on	Length of Time Tissue Was Heated in Boiling Saline Solution							
	5 sec.	10 sec.	15 sec.	20 sec.	25 sec.	30 sec.	40 sec.	45 sec.
Exterior	+	+		0		0		
Exterior	+	+		0		0		
Exterior	+	+		0		0		
Exterior	+	+		+		0		
Exterior	+	+		+		0		
Exterior	+	+		+		0		
Exterior	+	+		+		0		
Exterior		+		+		0		0
Exterior		+		+		0		0
Exterior		+		+		0		0
Exterior		+		+		0		0
Exterior		+		+		0	0	
Exterior		+		+		0	0	
Exterior		+		+		0	0	
Exterior		+		+		0	0	
Exterior and interior			+		+	0	0	
Exterior and interior			+		+	0	0	
Exterior and interior			+		+	0	0	
Exterior and interior			+		0	0	0	
Exterior and interior			+	+		0	0	
Exterior and interior			+	+		0	0	
Exterior and interior			+	+		0	0	
Exterior and interior			+	+		0	0	
Exterior and interior			+	+		0	0	
Exterior and interior			+	+		0	?	

* Staphylococcus aureus was used to inoculate the tissue.
+ = abundant growth; 0 = no colonies; ? = a few colonies.

from the heated tissue, a piece of tissue is dipped in a bacterial emulsion and then run through like the pieces which have been heated.

9. For deep tissue inoculation a hypodermic syringe is used, the bacterial emulsion being forced into the interior of the tissue and the whole then dipped in the same emulsion. The procedure from this point is the same as above. In this way the amount of heat necessary to destroy the surface bacteria without interfering with the organisms that are on the interior can be determined.

DISCUSSION

1. Boiling salt solution. The length of time required to destroy organisms on the surface of pieces of tissue dipped in suspensions of Staphylococcus aureus lies between 20 and 25 seconds, as growth was obtained after heating for 20 seconds in 12 of the series. In 3 series no growth occurred after heating 20 seconds. It made very little difference whether the tissue had been inoculated on the inside or the

TABLE 2

TESTS WITH BUNSEN FLAME

	Tissue Inoculated* on	Length of Time Tissue Was Kept in Bunsen Flame							
		1 sec.	3 sec.	5 sec.	10 sec.	20 sec.	25 sec.	30 sec.	40 sec.
I	Exterior	+	+	+	+?				
II	Exterior	+	+	+	+				
III	Exterior	+	+	+	+				
IV	Exterior	+	+	+	+				
V	Exterior	+	+	+	+				
VI	Exterior	+	+	+	+				
VII	Exterior	+	+	+	+				
VIII	Exterior	+	+	+	+				
IX	Exterior	+	+	+	+				
X	Exterior	+	+	+	+				
XI	Exterior				+	+		0	0
XII	Exterior				+	+		0	0
XIII	Exterior				+	+		0	0
XIV	Exterior				+	+		0	0
XV	Exterior				+	?		0	0
XVI	Exterior				+	+	0	0	
XVII	Exterior				+	0	0	0	
XVIII	Exterior				+	+	0	0	
XIX	Exterior				+	+	0	0	
XX	Exterior				+	+	0	0	
XXI	Exterior				+	?	0	0	
XXII	Exterior				+	+	0	0	
XXIII	Exterior				+	+	0	0	
XXIV	Exterior				+	?	0	0	
XXV	Exterior				+	+	0	0	
I	Exterior and interior				+	+	0	0	
II	Exterior and interior				+	+	0	0	
III	Exterior and interior				+	+	0	0	
IV	Exterior and interior				+	+	0	0	
V	Exterior and interior				+	+	0	0	
VI	Exterior and interior				+	+	0	0	
VII	Exterior and interior				+	+	0	0	
VIII	Exterior and interior				+	+	0	0	
IX	Exterior and interior				+	+	0	0	
X	Exterior and interior				+	+	0	0	

* Staphylococcus aureus was used to inoculate the tissue.
+ = abundant growth; 0 = no colonies; ? = a few colonies.

surface; in other words, if sufficient heat was applied to destroy surface bacteria, the organisms on the interior were also killed. The boiling salt solution method, then, leaves no margin of safety.

2. The results in Table 2 are even more surprising. There is growth after heating the tissue 20 seconds in a Bunsen flame above the cone, turning the tissue continually so that the flame will reach every

part. This is sufficient to char the tissue. In 10 series above 10 seconds was required, but not realizing that it would require such a length of time to destroy the organisms in the flame, no tests were made at longer than 10 seconds. In 15 other series the thermal death point was found to be above 20 seconds. This is sufficient to destroy the organisms on the interior as well. The only way that this can

TABLE 3

TESTS WITH LIQUID PARAFFIN

Series	Tissue Inoculated* on	Temp. of Oil	Length of Time Tissue Was Heated in Oil				
			2 sec.	5 sec.	10 sec.	15 sec.	20 sec.
I	Exterior	150 C.	+	+	+		
II	Exterior	150 C.	+	+	+		
III	Exterior	150 C.	+	+	+		
IV	Exterior	150 C.	+	+	+		
V	Exterior	180 C.		+	0	0	
VI	Exterior	180 C.		+	0	0	
VII	Exterior	180 C.		0	0	0	
VIII	Exterior	180 C.		+	0	0	
IX	Exterior	180 C.	+	+	0		
X	Exterior	180 C.	+	+	0		
XI	Exterior	180 C.	+	0	0		
XII	Exterior	180 C.	+	0	0		
XIII	Exterior	180 C.	+	0	0		
XIV	Exterior	180 C.	+	+	0		
XV	Exterior	190 C.	+	+	0		
XVI	Exterior	190 C.	+	0	0		
XVII	Exterior	190 C.	+	+	0		
XVIII	Exterior	190 C.	+	+	0		
XIX	Exterior	190 C.	+	0	0		
XX	Exterior	200 C.	0	0	0		
XXI	Exterior	200 C.	+	+?	0		
XXII	Exterior	200 C.	+	+?	0		
XXIII	Exterior	200 C.	+	0	0		
XXIV	Exterior	200 C.	+	0	0		
XXV	Exterior	200 C.	+	?	0		
I	Exterior and interior	180 C.		+	+		0
II	Exterior and interior	180 C.		+	+		0
III	Exterior and interior	180 C.		+	+		0
IV	Exterior and interior	180 C.		+	+		0
V	Exterior and interior	180 C.		+	+		0
VI	Exterior and interior	180 C.		+	+		0
VII	Exterior and interior	190 C.		+	+		0
VIII	Exterior and interior	190 C.		+	+		0
IX	Exterior and interior	190 C.		+	+		0
X	Exterior and interior	190 C.		+	+		0
XI	Exterior and interior	200 C.		+	0		0
XII	Exterior and interior	200 C.		+	0		0
XIII	Exterior and interior	200 C.		+	0		0
XIV	Exterior and interior	200 C.		+	0		0

* Staphylococcus aureus was used to inoculate the tissue.
+ = abundant growth; 0 = no colonies; ? = a few colonies.

be explained is by assuming that the charred tissue protects the bacteria, acting as a nonconductor of heat, or that the flame does not reach every part of the tissue.

When experiments with prostatic gland tissue were begun oil was considered a desirable medium. Its previous use was not known at that time, but it has since been learned that Fox[12] used oil in sterilizing tissues, as already mentioned.

3. In Table 3, where liquid paraffin was used at various temperatures and times, it was found that at 150 C. growth was still obtained at the end of 10 seconds in 4 series, so that the thermal death point for this temperature in these series was not definitely determined, while at 180 C. for 4 series the thermal death point was above 2 seconds but below 5 seconds, and for 6 series it was above 5 seconds and below 10 seconds. At 190 C. the thermal death point was practically the same as that at 180 C., while at 200 C. the thermal death point was below 2 seconds for 1 series, above 2 seconds for 2 series and above 5 seconds for 3 series. For the series where the organisms were

TABLE 4

THE GREATEST LENGTH OF TIME AT WHICH ANY GROWTH WAS OBTAINED IN EACH SERIES IS INDICATED

Boiling .085 percent. NaCl Solution

T. D. P. for Surface Organisms. Under		T. D. P. for Interior Organisms. Above		
20 seconds	30 seconds	15 sec.	20 sec.	25 sec.
3 series	12 series	1 series	6 series	3 series

Bunsen Flame

Undetermined*	20 seconds	25 seconds	30 seconds	20 seconds
10 series	1 series	9 series	5 series	10 series

Liquid Paraffin

Temp. of Paraffin	Below 2 seconds	2 seconds	5 seconds	10 seconds	5 seconds	10 seconds
150 C.	0	0	0	4 series		
180 C.	0	4 series	6 series	0	0	6 series
190 C.	0	2 series	3 series	0	0	4 series
200 C.	1 series	2 series	3 series	0	4 series	0

* In these ten series, the T. D. P. was somewhere above 10 seconds.

inoculated on the interior as well as on the exterior the thermal death point for 180 C. was above 10 seconds in 6 series, for 190 C. it was the same for 4 series and was found to be above 5 seconds for 4 series at 200 C.

The use of oil at 190 C. and 200 C. is accompanied by some inconvenience due to the ebullition of the oil at the time the tissue is introduced. This is not the case at 180 C. This series of experiments demonstrates that if a piece of tissue of the size used is dipped in oil at 180 C. for between 5 and 10 seconds, probably best at 6 seconds, bacteria on the surface will be destroyed without injuring the organisms

on the interior. It may be that if the tissue is obtained under rigid precautions and the outside washed several times in salt solution (as suggested by Bloomfield[7]) a shorter period would be allowable if one were dealing with a very thermo-sensitive organism on the interior of the tissue.

To procure pieces of tissue from the living body without contamination of the surface of these pieces is beyond doubt extraordinarily difficult. Great precautions must be taken in removing the tissue for the preparation of spirochaete culture media, where no other object is in view. In surgical operations, where searing of the skin, etc., cannot be practiced, the liability to contamination is increased. These considerations have guided previous bacteriologic workers in their efforts to sterilize the surface of the tissue, by methods which are here shown to be of doubtful value. In using the oil method, which is the best we have experimented with, one undoubtedly runs a risk of destroying delicate organisms within the tissue, although practically certain of freedom from surface contamination. But if any less effective method is used it is impossible to be sure that surface contamination has not influenced the results.

SUMMARY

With the air chamber and technic described in this paper danger of contamination from the outside during the making of cultures from tissues is reduced to the minimum.

It is apparent that the boiling water method as formerly used is inadequate for surface sterilization, and that the time required to sterilize the surface with certainty approaches that sufficient to sterilize the gland completely.

The same objections apply to the Bunsen flame. The flame either does not reach every part of the tissue, or the charred tissue acts as a nonconductor of heat.

The hot oil method of sterilization answers the purpose better than the preceding two methods, as at a temperature of 180 C., surface bacteria will be destroyed in 5 seconds without apparently interfering with the organisms in the interior of the tissue, which are not killed unless it is heated for more than 10 seconds.

In view of the stress laid on the bacteriology of glandular tissues in relation to the etiology of disease, it becomes apparent at once that surface sterilization is of the utmost importance. Having obtained a satisfactory method of surface sterilization, more confidence can be placed in the results obtained in future experiments.